

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
21 March 2002 (21.03.2002)

PCT

(10) International Publication Number
WO 02/22635 A1

(51) International Patent Classification⁷: **C07H 21/02**,
21/04, A61K 48/00, C12Q 1/68, C12P 19/34, C12N
15/85, 15/86

(21) International Application Number: PCT/US01/28235

(22) International Filing Date:
10 September 2001 (10.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/659,791 11 September 2000 (11.09.2000) US

(71) Applicant (*for all designated States except US*): **ISIS
PHARMACEUTICALS, INC.** [US/US]; 2292 Faraday
Avenue, Carlsbad, CA 92008 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **MONIA, Brett, P.**
[US/US]; 7605 Nueva Castilla Way, La Costa, CA 92009
(US). **FREIER, Susan, M.** [US/US]; 2946 Renault Street,
San Diego, CA 92122 (US).

(74) Agents: **LICATA, Jane, Massey et al.**; Licata & Tyrrell
P.C., 66 E. Main Street, Marlton, NJ 08053 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK,
SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA,
ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,
TG).

Published:

- *with international search report*
- *before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments*

*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: ANTISENSE MODULATION OF CLUSTERIN EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of clusterin. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding clusterin. Methods of using these compounds for modulation of clusterin expression and for treatment of diseases associated with expression of clusterin are provided.



WO 02/22635 A1

-1-

ANTISENSE MODULATION OF CLUSTERIN EXPRESSION

FIELD OF THE INVENTION

5 The present invention provides compositions and methods for modulating the expression of clusterin. In particular, this invention relates to compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding clusterin. Such compounds have
10 been shown to modulate the expression of clusterin.

BACKGROUND OF THE INVENTION

Clusterin is an amphipathic glycoprotein that was first isolated from the male reproductive system (Bettuzzi
15 et al., *Biochem. J.*, 1989, 257, 293-296; O'Bryan et al., *J. Clin. Invest.*, 1990, 85, 1477-1486). Subsequently, it has been shown that clusterin is ubiquitously distributed among tissues, having a wide range of biologic properties. Investigators from several disciplines, therefore, have
20 isolated clusterin homologs under more than ten different names reviewed in (Bailey and Griswold, *Mol. Cell. Endocrinol.*, 1999, 151, 17-23; Koch-Brandt and Morgans, *Prog. Mol. Subcell. Biol.*, 1996, 16, 130-149; Meri and Jarva, *Vox. Sang.*, 1998, 74, 291-302; Silkensen et al.,
25 *Biochem. Cell. Biol.*, 1994, 72, 483-488).

The clusterin protein consists of two non-identical subunits of 34 kDa and 47 kDa, designated alpha and beta, respectively. Clusterin expression is induced almost exclusively as a result of cellular injury, death, or
30 pathology.

Among its many roles, clusterin is a component of the soluble SCb-5 complement complex which is assembled in the plasma upon activation of the complement cascade (Choi et al., *Mol. Immunol.*, 1989, 26, 835-840; Kirszbaum et al.,
35 *Embo J.*, 1989, 8, 711-718; Murphy et al., *Int. Immunol.*,

1989, 1, 551-554; Tschopp and French, *Clin. Exp. Immunol.*,
1994, 97 Suppl 2, 11-14). Binding of clusterin has been
shown to abolish the membranolytic potential of complement
complexes and it has therefore been termed complement lysis
5 inhibitor (CLI) (Jenne and Tschopp, *Proc. Natl. Acad. Sci.*
U. S. A., 1989, 86, 7123-7127).

Further investigations of clusterin demonstrated that
it circulates in plasma as a high density lipoprotein (HDL)
complex which serves not only as an inhibitor of the lytic
10 complement cascade, but as a regulator of lipid transport
and local lipid redistribution (Jenne et al., *J. Biol.*
Chem., 1991, 266, 11030-11036). In this capacity,
clusterin isolated and characterized by de Silva et al. and
was given the name Apolipoprotein J (ApoJ) (de Silva et
15 al., *Biochemistry*, 1990, 29, 5380-5389; de Silva et al., *J.*
Biol. Chem., 1990, 265, 13240-13247; de Silva et al., *J.*
Biol. Chem., 1990, 265, 14292-14297). In these studies,
clusterin (ApoJ) was shown to play a role in cholesterol
transport in the liver and in the regulation of vascular
20 smooth muscle cell differentiation (de Silva et al., *J.*
Biol. Chem., 1990, 265, 13240-13247; Moulson and Millis, *J.*
Cell. Physiol., 1999, 180, 355-364). A link between the
modulation of HDL and complement activity is provided by
studies by James et al. that characterize the association
25 of a high density lipoprotein, NA1/NA2, with apolipoprotein
A-I (ApoA-I). This novel protein NA1/NA2, was subsequently
shown to be clusterin (James et al., *Arterioscler. Thromb.*,
1991, 11, 645-652).

Clusterin has also been shown to participate in the
30 cellular process of programmed cell death or apoptosis.
Clusterin expression demarcates cells undergoing apoptosis
(Buttayan et al., *Mol. Cell. Biol.*, 1989, 9, 3473-3481) and
in studies of the kidney, the onset of hydronephrosis
following unilateral obstruction is associated with the

increased expression of proteins encoded by the clusterin gene (Connor et al., *Kidney Int.*, 1991, 39, 1098-1103). In both of these studies, clusterin is referred to by two other synonyms, sulfated glycoprotein-2 gene (SGP-2) and
5 testosterone-repressed prostate message-2 (TRPM-2) (Buttayan et al., *Mol. Cell. Biol.*, 1989, 9, 3473-3481; Connor et al., *Kidney Int.*, 1991, 39, 1098-1103).

Sensibar et al. showed that cell death in the prostate, induced by tumor necrosis factor alpha, could be
10 prevented by overexpressing clusterin. In these studies, transfection of LNCaP cells with any of four 21-mer antisense phosphorothioate oligonucleotides targeting the clusterin coding region resulted in an increase of cell death (Sensibar et al., *Cancer Res.*, 1995, 55, 2431-2437).

15 Miyake et al. further demonstrated the role of clusterin as an anti-apoptotic gene in the Shionogi tumor model, a model used for the study of castration-induced apoptosis (Miyake et al., *Cancer Res.*, 2000, 60, 170-176).

In this model, androgen-dependent mammary carcinoma
20 xenograft tumors in male mice undergo regression after castration but recur as apoptosis-induced tumors after one month. Using a phosphorothioate 21-mer antisense oligonucleotide to the mouse clusterin gene targeting the translation initiation site, Miyake et al. were able to
25 show that treatment with the clusterin antisense oligonucleotide of mice with Shionogi tumors resulted in a more rapid onset of apoptosis and time to complete regresssion. There was also a significant delay of emergence of androgen-independent recurrent tumors compared
30 to control oligonucleotide treated controls.

Using the same oligonucleotide in an experiment designed to test the efficacy of the oligonucleotide in combination with paclitaxel, Miyake et al. showed that the combination of antisense oligonucleotide and paclitaxel
35 induced apoptosis in Shionogi tumors better than either

agent alone. These studies suggest that the antisense oligonucleotide may be useful in enhancing the effects of cytotoxic chemotherapy in hormone-refractory prostate cancer (Miyake et al., *Cancer Res.*, 2000, 60, 2547-2554).

5 Ten antisense oligodeoxynucleotides targeted to human TRPM-2 (clusterin) were designed by Miyake et al. (*Clin. Cancer Res.*, 2000, 6, 1655-1663) to identify potent oligonucleotides that specifically inhibit TRPM-2 expression in human androgen-independent prostate cancer
10 PC-2 cells. Seven of the ten oligonucleotides had little or no effect on TRPM-2 mRNA expression. The other three oligonucleotides were described by the authors as having moderate effects. The most active oligonucleotide was also tested for ability to enhance the response of PC-3 cells to
15 Taxol or mitoxantrone.

Another antisense oligonucleotide, targeting the AUG initiation codon of clusterin was used to investigate the role of clusterin in endothelial cell activation. In these studies, it was shown that clusterin expression is
20 upregulated upon laminar shear stress and that reduction of clusterin levels via antisense treatment increased endothelial cell activation (Urbich et al., *Circulation*, 2000, 101, 352-355).

The level of clusterin is increased in the hippocampus
25 and frontal cortex of the brains of Alzheimer's disease patients. It is currently believed that clusterin, by binding to beta-amyloid, a protein known to aggregate in the brains of these patients, acts to link the progression of this disease to the complement system (Choi-Miura and
30 Oda, *Neurobiol. Aging*, 1996, 17, 717-722).

Most recently, clusterin has been isolated as a KU70 binding protein. KU binding proteins (KUBs) are involved in DNA repair pathways. Clusterin (KUB1) was identified as an autoantigen in serum of patients with scleroderma-
35 polymyositis syndrome and shown to dimerize with KUP80 to

form an ATP dependent helicase and a regulatory component of a DNA dependent protein kinase (PRKDC) involved in double-strand break repair and V(D)J recombination (Yang et al., *Nucleic Acids Res.*, 1999, 27, 2165-2174).

5 Clusterin is overexpressed in many disease states including neurodegenerative disorders, gliomas, retinitis pigmentosa and expression is induced in acute and chronic models of renal injury and disease, following ureter obstruction, ischemia/reperfusion, and atherosclerosis
10 reviewed in (Silkensen et al., *Biochem. Cell. Biol.*, 1994, 72, 483-488). The pharmacological modulation of clusterin activity and/or expression may therefore be an appropriate point of therapeutic intervention in pathological conditions.

15 The expression of clusterin, or variants thereof, has been used as a means of differentiating normal versus abnormal cells in the study of male infertility. A method of assessing acrosomal status of sperm morphology comprising contacting a sperm sample with an
20 immunologically reactive molecule which binds to one form of clusterin and not another is disclosed in WO 95/16916.

Currently, there are no known therapeutic agents which effectively inhibit the synthesis of clusterin and to date, investigative strategies aimed at modulating clusterin
25 function have involved the use of antibodies, antisense oligonucleotides and chemical inhibitors.

There remains, however, a long felt need for additional agents capable of effectively inhibiting clusterin function.

30 Antisense technology is emerging as an effective means for reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of clusterin expression.

The present invention provides compositions and methods for modulating clusterin expression, including modulation of the alpha and/or beta subunits.

5 SUMMARY OF THE INVENTION

The present invention is directed to compounds, particularly antisense oligonucleotides, which are targeted to a nucleic acid encoding clusterin, and which modulate the expression of clusterin. Pharmaceutical and other
10 compositions comprising the compounds of the invention are also provided. Further provided are methods of modulating the expression of clusterin in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention.
15 Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of clusterin by administering a therapeutically or prophylactically effective amount of one or more of the
20 antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in
25 modulating the function of nucleic acid molecules encoding clusterin, ultimately modulating the amount of clusterin produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding clusterin. As used herein, the
30 terms "target nucleic acid" and "nucleic acid encoding clusterin" encompass DNA encoding clusterin, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes
35 with the normal function of the nucleic acid. This

modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of clusterin. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding clusterin. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the

gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding clusterin, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous

nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It

has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified,
5 oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization"
10 means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds.
15 "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide
20 and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen
25 bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is
30 understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule
35 interferes with the normal function of the target DNA or

RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e.,
5 under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed.

Antisense and other compounds of the invention which
10 hybridize to the target and inhibit expression of the target are identified through experimentation, and the sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The target sites to which these preferred sequences are complementary are
15 hereinbelow referred to as "active sites" and are therefore preferred sites for targeting. Therefore another embodiment of the invention encompasses compounds which hybridize to these active sites.

Antisense compounds are commonly used as research
20 reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to
25 distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic
30 uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are
35 presently underway. It is thus established that

-12-

oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (i.e. from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further

include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and

those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

5 Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 10 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

15 Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short 20 chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; 25 methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

30 Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 35 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289;

5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

5 In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such
10 oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular
15 an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited
20 to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, 1991, 254, 1497-1500.

Most preferred embodiments of the invention are
25 oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-\text{CH}_2-\text{NH}-\text{O}-\text{CH}_2-$, $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{O}-\text{CH}_2-$ [known as a methylene (methylimino) or MMI backbone], $-\text{CH}_2-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-$, $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{N}(\text{CH}_3)-\text{CH}_2-$ and $-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-$ [wherein the
30 native phosphodiester backbone is represented as $-\text{O}-\text{P}-\text{O}-\text{CH}_2-$] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S.
35 patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH₂-O-CH₂-N(CH₂)₂, also described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in

-17-

2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents
5 that teach the preparation of such modified sugar structures include, but are not limited to, U.S.:
4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878;
5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811;
5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053;
10 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often
15 referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other
20 synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-
25 thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl
30 and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No.
35 3,687,808, those disclosed in *The Concise Encyclopedia Of*

Polymer Science And Engineering, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B. , ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which

enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 5 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et 10 al., *Nucl. Acids Res.*, 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, 1990, 259, 327-330; Svinarchuk et al., *Biochimie*, 1993, 75, 49-54), a phospholipid, e.g., di- 15 hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & 20 Nucleotides*, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. 25 Pharmacol. Exp. Ther.*, 1996, 277, 923-937).

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 30 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830;

5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506;
5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241,
5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667;
5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481;
5 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and
5,688,941, certain of which are commonly owned with the
instant application, and each of which is herein
incorporated by reference.

It is not necessary for all positions in a given
10 compound to be uniformly modified, and in fact more than
one of the aforementioned modifications may be incorporated
in a single compound or even at a single nucleoside within
an oligonucleotide. The present invention also includes
antisense compounds which are chimeric compounds.
15 "Chimeric" antisense compounds or "chimeras," in the
context of this invention, are antisense compounds,
particularly oligonucleotides, which contain two or more
chemically distinct regions, each made up of at least one
monomer unit, i.e., a nucleotide in the case of an
20 oligonucleotide compound. These oligonucleotides typically
contain at least one region wherein the oligonucleotide is
modified so as to confer upon the oligonucleotide increased
resistance to nuclease degradation, increased cellular
uptake, and/or increased binding affinity for the target
25 nucleic acid. An additional region of the oligonucleotide
may serve as a substrate for enzymes capable of cleaving
RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is
a cellular endonuclease which cleaves the RNA strand of an
RNA:DNA duplex. Activation of RNase H, therefore, results
30 in cleavage of the RNA target, thereby greatly enhancing
the efficiency of oligonucleotide inhibition of gene
expression. Consequently, comparable results can often be
obtained with shorter oligonucleotides when chimeric
oligonucleotides are used, compared to phosphorothioate
35 deoxyoligonucleotides hybridizing to the same target

region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

5 Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to
10 in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.:
5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878;
5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355;
15 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through
20 the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well
25 known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector
30 constructs designed to direct the in vivo synthesis of antisense molecules.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as
35 for example, liposomes, receptor targeted molecules, oral,

rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting
5 formulations include, but are not limited to, U.S.:
5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291;
5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330;
4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170;
5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978;
10 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259;
5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such
15 esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically
20 acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an
25 active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to
30 the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 and U.S. 5,770,713 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of
35 the compounds of the invention: i.e., salts that retain the

-23-

desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid,

succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid,

tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed
5 from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an
10 animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of clusterin is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in
15 pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection,
20 inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding clusterin, enabling sandwich and other assays to easily be constructed to
25 exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding clusterin can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide
30 or any other suitable detection means. Kits using such detection means for detecting the level of clusterin in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense
35 compounds of the invention. The pharmaceutical

compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and

liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

5 The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association
10 the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the
15 product.

 The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the
20 present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The
25 suspension may also contain stabilizers.

 In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams,
30 jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may

be applied to the formulation of the compositions of the present invention.

Emulsions

5 The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. (Idson, in *Pharmaceutical Dosage Forms*,
10 Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman,
15 Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases
20 intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a
25 water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to
30 the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed.

Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex
5 formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water
10 stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into
15 the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing
20 emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in
25 *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of
30 emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc.,
35 New York, N.Y., 1988, volume 1, p. 199). Surfactants are

typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool
5 in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*,
10 Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic
15 properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous
20 preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as
25 carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants,
30 hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel
35 Dekker, Inc., New York, N.Y., volume 1, p. 199).

-31-

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth),
5 cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming
10 strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides
15 that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic
20 acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing
25 agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for
30 their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of
35 ease of formulation, efficacy from an absorption and

bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

10 In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules

(Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a
5 comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger
10 and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed
15 spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate
20 (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with
25 cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant
30 molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300,
35 PEG400, polyglycerols, propylene glycols, and derivatives

of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty
5 acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced
10 absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides
(Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*,
15 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation,
20 ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity
(Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385; Ho et al., *J. Pharm. Sci.*, 1996, 85, 138-143). Often microemulsions may form spontaneously when their components
25 are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical
30 applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local
35 cellular uptake of oligonucleotides and nucleic acids

within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes has been discussed above.

Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable
5 to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of
10 water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245).
15 Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the
20 liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the
25 active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over
30 other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and
35 hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., *Biochem. Biophys. Res. Commun.*, 1987, 147, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., *Journal of Controlled Release*, 1992, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean

PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of
5 liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an emulsion) were ineffective (Weiner et al., *Journal of Drug Targeting*, 1992, 2, 405-410). Further, an additional study
10 tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis et al., *Antiviral Research*, 1992, 18, 259-265).

15 Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl
20 dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems
25 were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. *S.T.P. Pharma. Sci.*, 1994, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to
30 liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming

lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1} , or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen et al., *FEBS Letters*, 1987, 223, 42; Wu et al., *Cancer Research*, 1993, 53, 3765). Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos et al. (*Ann. N.Y. Acad. Sci.*, 1987, 507, 64) reported the ability of monosialoganglioside G_{M1} , galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon et al. (*Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim et al.).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (*Bull. Chem. Soc. Jpn.*, 1980, 53, 2778) described liposomes comprising a nonionic detergent, 2C₁₂15G, that contains a PEG moiety. Illum et al. (*FEBS Lett.*, 1984, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood

half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov et al. (*FEBS Lett.*, 1990, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume et al. (*Biochimica et Biophysica Acta*, 1990, 1029, 91) extended such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle et al. (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin et al. (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin et al.) and in WO 94/20073 (Zalipsky et al.) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi et al.). U.S. Patent Nos. 5,540,935 (Miyazaki et al.) and 5,556,948 (Tagawa et al.) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry et al. discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa et al. discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman et al.

describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love et al. discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

5 Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through
10 pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, e.g. they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To
15 make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as
20 subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties
25 of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations
30 (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic
35 products and are usable over a wide range of pH values. In

general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan
5 esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of
10 the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of
15 amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl
20 sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The
25 quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include
30 acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in
Pharmaceutical Dosage Forms, Marcel Dekker, Inc., New York,
35 NY, 1988, p. 285).

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient
5 delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic
10 drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

15 Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92). Each of the above
20 mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical
25 entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In
30 addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92); and perfluorochemical

emulsions, such as FC-43. Takahashi et al., *J. Pharm. Pharmacol.*, 1988, 40, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example,
5 oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-
10 dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C₁₋₁₀ alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in*
15 *Therapeutic Drug Carrier Systems*, 1991, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; El Hariri et al., *J. Pharm. Pharmacol.*, 1992, 44, 651-654).

20 Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pp.
25 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for
30 example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium

glycodeoxycholate), taurocholic acid (sodium taurocholate),
taurodeoxycholic acid (sodium taurodeoxycholate),
chenodeoxycholic acid (sodium chenodeoxycholate),
ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-
5 fusidate (STDHF), sodium glycodihydrofusidate and
polyoxyethylene-9-lauryl ether (POE) (Lee et al., *Critical
Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92;
Swinyard, Chapter 39 In: *Remington's Pharmaceutical
Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co.,
10 Easton, PA, 1990, pages 782-783; Muranishi, *Critical
Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33;
Yamamoto et al., *J. Pharm. Exp. Ther.*, 1992, 263, 25;
Yamashita et al., *J. Pharm. Sci.*, 1990, 79, 579-583).

15 Chelating Agents: Chelating agents, as used in
connection with the present invention, can be defined as
compounds that remove metallic ions from solution by
forming complexes therewith, with the result that
absorption of oligonucleotides through the mucosa is
20 enhanced. With regards to their use as penetration
enhancers in the present invention, chelating agents have
the added advantage of also serving as DNase inhibitors, as
most characterized DNA nucleases require a divalent metal
ion for catalysis and are thus inhibited by chelating
25 agents (Jarrett, *J. Chromatogr.*, 1993, 618, 315-339).
Chelating agents of the invention include but are not
limited to disodium ethylenediaminetetraacetate (EDTA),
citric acid, salicylates (e.g., sodium salicylate, 5-
methoxysalicylate and homovanilate), N-acyl derivatives of
30 collagen, laureth-9 and N-amino acyl derivatives of beta-
diketones (enamines) (Lee et al., *Critical Reviews in
Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi,
Critical Reviews in Therapeutic Drug Carrier Systems, 1990,
7, 1-33; Buur et al., *J. Control Rel.*, 1990, 14, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, 1987, 39, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

30 Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized

as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation.

5 The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition
10 between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-
15 4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., *Antisense Res. Dev.*, 1995, 5, 115-121; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, 1996, 6, 177-183).

Excipients

20 In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is
25 selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to,
30 binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen
35 phosphate, etc.); lubricants (e.g., magnesium stearate,

talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

30

Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the

35

compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include, but are not limited to, anticancer drugs such as daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil (5-FU), floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds.,

1987, Rahway, N.J., pages 1206-1228). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50} s found to be effective in in vitro and in vivo animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years.

- 51 -

Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be
5 desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

10 While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

15

EXAMPLES

Example 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis Deoxy and 2'-alkoxy amidites

20 2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein
25 incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

30 Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, 1993, 21, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes,
35 Needham MA).

2'-Fluoro amidites**2'-Fluorodeoxyadenosine amidites**

2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, 1993, 36, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was

-53-

accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to

give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or
5 it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

10 2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the
15 solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH₃CN (600 mL) and evaporated. A silica
20 gel column (3 kg) was packed in CH₂Cl₂/acetone/MeOH (20:5:3) containing 0.5% Et₃NH. The residue was dissolved in CH₂Cl₂ (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional
25 material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue
30 dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one

-55-

hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH₃CN (200 mL). The residue was dissolved in CHCl₃ (1.5 L) and extracted with 2x500 mL of saturated NaHCO₃ and 2x500 mL of saturated NaCl. The organic phase was dried over Na₂SO₄, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et₃NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

15 **3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the addition of MeOH. Upon completion of the reaction, as judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in CHCl₃ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl₃. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH₃CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH₃CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl₃ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO₃ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH₄OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL).

The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH₃ gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with

saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

5 **N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with
10 stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotrope with MeOH (200 mL). The residue was dissolved in CHCl₃ (700 mL) and extracted with saturated NaHCO₃ (2x300 mL) and saturated NaCl (2x300
15 mL), dried over MgSO₄ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et₃NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

20

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH₂Cl₂ (1 L).
25 Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction
30 mixture was extracted with saturated NaHCO₃ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH₂Cl₂ (300 mL), and the extracts were combined, dried over MgSO₄ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column
35 using EtOAc/hexane (3:1) as the eluting solvent. The pure

fractions were combined to give 90.6 g (87%) of the title compound.

2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-
5 (dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also
known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside
10 amidites] are prepared as described in the following
paragraphs. Adenosine, cytidine and guanosine nucleoside
amidites are prepared similarly to the thymidine (5-
methyluridine) except the exocyclic amines are protected
with a benzoyl moiety in the case of adenosine and cytidine
15 and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O²-2'-anhydro-5-
methyluridine

O²-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese,
20 Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g,
0.013eq, 0.0054mmol) were dissolved in dry pyridine (500
ml) at ambient temperature under an argon atmosphere and
with mechanical stirring. tert-Butyldiphenylchlorosilane
(125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one
25 portion. The reaction was stirred for 16 h at ambient
temperature. TLC (R_f 0.22, ethyl acetate) indicated a
complete reaction. The solution was concentrated under
reduced pressure to a thick oil. This was partitioned
between dichloromethane (1 L) and saturated sodium
30 bicarbonate (2x1 L) and brine (1 L). The organic layer was
dried over sodium sulfate and concentrated under reduced
pressure to a thick oil. The oil was dissolved in a 1:1
mixture of ethyl acetate and ethyl ether (600mL) and the
solution was cooled to

-10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-0²-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting

- 60 -

material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-

5 5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried
10 over P₂O₅ under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The
15 rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was
20 evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

25

5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH₂Cl₂
30 (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CH₂Cl₂ and the combined organic phase was washed with water, brine and dried over anhydrous Na₂SO₄. The solution was concentrated

-61-

to get 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%).

10 **5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine**

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH₂Cl₂). Aqueous NaHCO₃ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na₂SO₄, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% NaHCO₃ (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue obtained was

-62-

purified by flash column chromatography and eluted with 5% MeOH in CH₂Cl₂ to get 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

5

2'-O-(dimethylaminoxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was
10 then added to 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH₂Cl₂). Solvent was removed under vacuum and the residue placed on a flash column and
15 eluted with 10% MeOH in CH₂Cl₂ to get 2'-O-(dimethylaminoxyethyl)-5-methyluridine (766mg, 92.5%).

5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine

2'-O-(dimethylaminoxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P₂O₅ under high vacuum overnight at
20 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture
25 was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH₂Cl₂ (containing a few drops of pyridine) to get 5'-O-DMT-
30 2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminoxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine
5 (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P₂O₅ under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-
10 N,N,N¹,N¹-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was
15 dissolved in ethyl acetate (70mL) and washed with 5% aqueous NaHCO₃ (40mL). Ethyl acetate layer was dried over anhydrous Na₂SO₄ and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminoxyethyl)-5-methyluridine-3'-[(2-
20 cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites [also known in
25 the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

30 N2-isobutyryl-6-O-diphenylcarbamoyle-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine

- 64 -

riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH₂-O-CH₂-N(CH₂)₂, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. 0²-, 2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium

-65-

bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned
5 between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The
10 residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

15

5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine in anhydrous pyridine (8
20 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH₂Cl₂ (2x200 mL). The combined CH₂Cl₂ layers are washed with saturated NaHCO₃ solution, followed by
25 saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using MeOH:CH₂Cl₂:Et₃N (20:1, v/v, with 1% triethylamine) gives the title compound.

30

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added

-66-

to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH_2Cl_2 (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

Example 2

10 Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

15 Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent

5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, 5 respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as 10 described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

15

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedi- 20 methylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleo- 25 sides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

30 Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by 35 reference.

Example 4**PNA Synthesis**

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, 1996, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5**Synthesis of Chimeric Oligonucleotides**

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me] -- [2'-deoxy] -- [2'-O-Me] Chimeric

Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above.

Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The

- 69 -

standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl)Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester)]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidization with iodine to generate

the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,4-dihydro-2H-benzodithiole-3-one 1,1-dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

10

Example 6

Oligonucleotide Isolation

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ³¹P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* 1991, 266, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

30 Example 7

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences

-71-

simultaneously in a standard 96 well format.

Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate

internucleotide linkages were generated by sulfurization

5 utilizing 3,4-dithiolane-2-one 1,1-dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ).

10 Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature
15 (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

20

Example 8

Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption
25 spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone
30 composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate

were at least 85% full length.

Example 9

Cell culture and oligonucleotide treatment

5 The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following
10 4 cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, Ribonuclease protection
15 assays, or RT-PCR.

T-24 cells:

 The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture
20 Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and
25 streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for
30 use in RT-PCR analysis.

 For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

-73-

A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

30 Treatment with antisense compounds:

When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200 μ L OPTI-MEMTM-1 reduced-serum medium (Gibco BRL) and then treated with 130 μ L of

OPTI-MEM™-1 containing 3.75 µg/mL LIPOFECTIN™ (Gibco BRL) and the desired concentration of oligonucleotide. After 4-7 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16-24 hours after
5 oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control
10 oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is ISIS 13920, **TCCGTCATCGCTCCTCAGGG**, SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras.
15 For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCATTCTGCCCCCAAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive
20 control oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest
25 concentration of positive control oligonucleotide that results in 60% inhibition of H-ras or c-raf mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is
30 deemed as unsuitable for oligonucleotide transfection experiments.

Example 10**Analysis of oligonucleotide inhibition of clusterin expression**

Antisense modulation of clusterin expression can be
5 assayed in a variety of ways known in the art. For
example, clusterin mRNA levels can be quantitated by, e.g.,
Northern blot analysis, competitive polymerase chain
reaction (PCR), or real-time PCR (RT-PCR). Real-time
quantitative PCR is presently preferred. RNA analysis can
10 be performed on total cellular RNA or poly(A)+ mRNA.
Methods of RNA isolation are taught in, for example,
Ausubel, F.M. et al., *Current Protocols in Molecular
Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John
Wiley & Sons, Inc., 1993. Northern blot analysis is
15 routine in the art and is taught in, for example, Ausubel,
F.M. et al., *Current Protocols in Molecular Biology*, Volume
1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-
time quantitative (PCR) can be conveniently accomplished
using the commercially available ABI PRISM™ 7700 Sequence
20 Detection System, available from PE-Applied Biosystems,
Foster City, CA and used according to manufacturer's
instructions. Prior to quantitative PCR analysis, primer-
probe sets specific to the target gene being measured are
evaluated for their ability to be "multiplexed" with a
25 GAPDH amplification reaction. In multiplexing, both the
target gene and the internal standard gene GAPDH are
amplified concurrently in a single sample. In this
analysis, mRNA isolated from untreated cells is serially
diluted. Each dilution is amplified in the presence of
30 primer-probe sets specific for GAPDH only, target gene only
("single-plexing"), or both (multiplexing). Following PCR
amplification, standard curves of GAPDH and target mRNA
signal as a function of dilution are generated from both
the single-plexed and multiplexed samples. If both the

slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed as multiplexable. Other methods of PCR are also known in the art.

Protein levels of clusterin can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to clusterin can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., 1997.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., 1998. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., 1997. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

Example 11**Poly(A)+ mRNA isolation**

Poly(A)+ mRNA was isolated according to Miura et al.,
5 Clin. Chem., 1996, 42, 1758-1764. Other methods for
poly(A)+ mRNA isolation are taught in, for example,
Ausubel, F.M. et al., *Current Protocols in Molecular
Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons,
Inc., 1993. Briefly, for cells grown on 96-well plates,
10 growth medium was removed from the cells and each well was
washed with 200 μ L cold PBS. 60 μ L lysis buffer (10 mM Tris-
HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM
vanadyl-ribonucleoside complex) was added to each well, the
plate was gently agitated and then incubated at room
15 temperature for five minutes. 55 μ L of lysate was
transferred to Oligo d(T) coated 96-well plates (AGCT Inc.,
Irvine CA). Plates were incubated for 60 minutes at room
temperature, washed 3 times with 200 μ L of wash buffer (10
mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the
20 final wash, the plate was blotted on paper towels to remove
excess wash buffer and then air-dried for 5 minutes. 60 μ L
of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C
was added to each well, the plate was incubated on a 90°C
hot plate for 5 minutes, and the eluate was then
25 transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be
treated similarly, using appropriate volumes of all
solutions.

30 Example 12**Total RNA Isolation**

Total mRNA was isolated using an RNEASY 96™ kit and
buffers purchased from Qiagen Inc. (Valencia CA) following

-78-

the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ L cold PBS. 100 μ L Buffer RLT was added to each well and the plate
5 vigorously agitated for 20 seconds. 100 μ L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96TM well plate attached to a QIAVACTM manifold fitted with a waste collection tray and
10 attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96TM plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96TM plate and the vacuum applied for a period of
15 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVACTM manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVACTM manifold fitted with a collection tube rack
20 containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 μ L water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 μ L water.

The repetitive pipetting and elution steps may be
25 automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

Example 13**Real-time Quantitative PCR Analysis of clusterin mRNA Levels**

Quantitation of clusterin mRNA levels was determined
5 by real-time quantitative PCR using the ABI PRISM™ 7700
Sequence Detection System (PE-Applied Biosystems, Foster
City, CA) according to manufacturer's instructions. This
is a closed-tube, non-gel-based, fluorescence detection
system which allows high-throughput quantitation of
10 polymerase chain reaction (PCR) products in real-time. As
opposed to standard PCR, in which amplification products
are quantitated after the PCR is completed, products in
real-time quantitative PCR are quantitated as they
accumulate. This is accomplished by including in the PCR
15 reaction an oligonucleotide probe that anneals specifically
between the forward and reverse PCR primers, and contains
two fluorescent dyes. A reporter dye (e.g., JOE, FAM, or
VIC, obtained from either Operon Technologies Inc.,
Alameda, CA or PE-Applied Biosystems, Foster City, CA) is
20 attached to the 5' end of the probe and a quencher dye
(e.g., TAMRA, obtained from either Operon Technologies
Inc., Alameda, CA or PE-Applied Biosystems, Foster City,
CA) is attached to the 3' end of the probe. When the probe
and dyes are intact, reporter dye emission is quenched by
25 the proximity of the 3' quencher dye. During
amplification, annealing of the probe to the target
sequence creates a substrate that can be cleaved by the 5'-
exonuclease activity of Taq polymerase. During the
extension phase of the PCR amplification cycle, cleavage of
30 the probe by Taq polymerase releases the reporter dye from
the remainder of the probe (and hence from the quencher
moiety) and a sequence-specific fluorescent signal is
generated. With each cycle, additional reporter dye
molecules are cleaved from their respective probes, and the
35 fluorescence intensity is monitored at regular intervals by

laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM MgCl₂, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD™, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 µL poly(A) mRNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

Probes and primers to human clusterin were designed to hybridize to a human clusterin sequence, using published sequence information (GenBank accession number M64722, incorporated herein as SEQ ID NO:3). For human clusterin the PCR primers were:

forward primer: TCCGTACGAGCCCCTGAA (SEQ ID NO: 4)
reverse primer: TGAGCCTCGTGTATCATCTCAAG (SEQ ID NO: 5) and
the PCR probe was: FAM-TCCACGCCATGTTCCAGCCCT-TAMRA (SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For human GAPDH the PCR primers were:
forward primer: CAACGGATTTGGTCGTATTGG (SEQ ID NO: 7)
reverse primer: GGCAACAATATCCACTTTACCAGAGT (SEQ ID NO: 8)

-81-

and the PCR probe was: 5' JOE-CGCCTGGTCACCAGGGCTGCT- TAMRA
3' (SEQ ID NO: 9) where JOE (PE-Applied Biosystems, Foster
City, CA) is the fluorescent reporter dye) and TAMRA (PE-
Applied Biosystems, Foster City, CA) is the quencher dye.

5

Example 14

Northern blot analysis of clusterin mRNA levels

Eighteen hours after antisense treatment, cell
monolayers were washed twice with cold PBS and lysed in 1
10 mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA
was prepared following manufacturer's recommended
protocols. Twenty micrograms of total RNA was fractionated
by electrophoresis through 1.2% agarose gels containing
1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc.
15 Solon, OH). RNA was transferred from the gel to HYBOND™-N+
nylon membranes (Amersham Pharmacia Biotech, Piscataway,
NJ) by overnight capillary transfer using a
Northern/Southern Transfer buffer system (TEL-TEST "B"
Inc., Friendswood, TX). RNA transfer was confirmed by UV
20 visualization. Membranes were fixed by UV cross-linking
using a STRATALINKER™ UV Crosslinker 2400 (Stratagene,
Inc, La Jolla, CA) and then probed using QUICKHYB™
hybridization solution (Stratagene, La Jolla, CA) using
manufacturer's recommendations for stringent conditions.

25 To detect human clusterin, a human clusterin specific
probe was prepared by PCR using the forward primer
TCCGTACGAGCCCCTGAA (SEQ ID NO: 4) and the reverse primer
TGAGCCTCGTGTATCATCTCAAG (SEQ ID NO: 5). To normalize for
variations in loading and transfer efficiency membranes
30 were stripped and probed for human glyceraldehyde-3-
phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto,
CA).

Hybridized membranes were visualized and quantitated
using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3

(Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

Example 15

5 Antisense inhibition of human clusterin expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

In accordance with the present invention, a series of oligonucleotides were designed to target different regions
10 of the human clusterin RNA, using published sequences (GenBank accession number M64722, incorporated herein as SEQ ID NO: 3, GenBank accession number L00974, incorporated herein as SEQ ID NO: 10, GenBank accession number M63377, incorporated herein as SEQ ID NO: 11, GenBank accession
15 number M63376, incorporated herein as SEQ ID NO: 12, and GenBank accession number M25915, incorporated herein as SEQ ID NO: 13). The oligonucleotides are shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the
20 oligonucleotide binds. All compounds in Table 1 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings
25 are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human clusterin mRNA levels by
30 quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 1

Inhibition of human clusterin mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

5

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	%INHIB	SEQ ID NO
129045	5'UTR	3	18	gtctttgcacgcctcggtca	64	14
129046	5'UTR	3	26	attctggagtcctttgcacgc	64	15
129047	Start Codon	3	44	gtcttcatcatgcctccaat	68	16
129048	Coding	3	82	tctcccaggtcagcagcagc	67	17
129049	Coding	3	106	tctggtcccccaggacctgc	46	18
129050	Coding	3	127	ggagctcattgtctgagacc	59	19
129052	Coding	3	154	acttacttccctgattggac	77	20
129053	Coding	3	171	aatttccttattgacgtact	68	21
129054	Coding	3	206	gtctttatctgtttcacccc	85	22
129055	Coding	3	286	gggcatcctctttcttcttc	64	23
129056	Coding	3	291	atttagggcatcctctttct	31	24
129057	Coding	3	303	ttccctgggtctcatttaggg	68	25
129058	Coding	3	312	tgtctctgattccctgggtct	79	26
129059	Coding	3	329	gggagctccttcagctttgt	49	27
129060	Coding	3	364	cccagagggccatcatggtc	45	28
129061	Coding	3	369	ctcttcccagagggccatca	36	29
129062	Coding	3	385	tcaggcagggcttacactct	70	30
129063	Coding	3	412	gtgcgtagaacttcatgcag	60	31
129064	Coding	3	448	ggcggccaaccaggcctgag	42	32
129065	Coding	3	449	tggcggccaaccaggcctga	32	33
129066	Coding	3	460	actcctcaagctggcggcca	67	34
129067	Coding	3	487	agtagaagggcgagctctgg	63	35
129068	Coding	3	497	ttcatccagaagtagaaggg	41	36
129069	Coding	3	522	cagcagggagtcgatgcggt	60	37
129070	Coding	3	538	gctgccggtcgttctccagc	51	38
129071	Coding	3	556	catccagcatgtgcgtctgc	69	39
129072	Coding	3	558	gacatccagcatgtgcgtct	55	40
129073	Coding	3	570	gtggtcctgcatgacatcca	62	41
129074	Coding	3	572	aagtggctcctgcatgacatc	41	42
129075	Coding	3	609	ctggaagagctcgtctatga	67	43
129076	Coding	3	613	tgtcctggaagagctcgtct	69	44
129077	Coding	3	618	gaacctgtcctggaagagct	68	45
129078	Coding	3	695	ggaaagaagaagtgaggcct	44	46
129079	Coding	3	726	gggcatcaagctgcggacga	65	47
129080	Coding	3	780	ctcaaggaagggtggaaca	81	48
129081	Coding	3	781	tctcaaggaagggtggaac	81	49
129082	Coding	3	788	tgtatcatctcaaggaaggg	38	50
129083	Coding	3	825	gctgtggaagtggatgtcca	48	51
129084	Coding	3	853	attctgttggcgggtgctgg	50	52
129085	Coding	3	858	tatgaattctgttggcgggt	36	53
129086	Coding	3	898	ggatctcccggcacacagtc	68	54
129087	Coding	3	899	cggatctcccggcacacagt	70	55

129088	Coding	3	911	gtggagttgtggcggatctc	69	56
129089	Coding	3	933	gtccttcatccgcaggcagc	56	57
129090	Coding	3	972	acagtccacagacaagatct	49	58
129092	Coding	3	1014	gagctcccgcgcagcttag	22	59
129093	Coding	3	1027	ggagggattcgtcgagctcc	55	60
129094	Coding	3	1088	atcttccactggtaggactt	50	61
129095	Coding	3	1096	tgttgagcatcttccactgg	62	62
129096	Coding	3	1118	agctgctccagcaaggagga	46	63
129097	Coding	3	1126	gctcgttcagctgctccagc	43	64
129098	Coding	3	1153	ttgccagccgggacacccag	72	65
129099	Coding	3	1187	cgcagatagtactggtcttc	61	66
129100	Coding	3	1199	accgtggtgacccgcagata	73	67
129101	Coding	3	1221	cgagtcagaagtgtgggaag	24	68
129102	Coding	3	1280	gtgatgggatcagagtcaaa	38	69
129103	Coding	3	1305	ggagacttctacagggaccg	63	70
129104	Coding	3	1337	gccacggtctccataaattt	70	71
129106	3'UTR	3	1403	gcaaaagcaacatccacatc	74	72
129107	3'UTR	3	1550	tagagtgcaggatccagagc	71	73
129108	3'UTR	3	1605	attagttgcatgcaggagca	71	74
129109	3'UTR	3	1620	agacagttttattgaattag	11	75
129118	Intron	10	2819	cgagatagagccactgtacg	44	76
129119	Intron	10	4646	tgccaccacccccgggtgat	13	77
129091	Intron- Exon Junction	10	5849	gttggtggtggaacagtcca	40	78
129120	Intron- Exon Junction	10	7384	tgcttaccggtgctttttgc	46	79
129105	Intron- Exon Junction	10	7600	acatctcactcctcccggtg	70	80
129121	3'UTR	10	7855	gaccctccaagcgatcagct	20	81
129122	3'UTR	10	7863	aaaaagaggaccctccaagc	39	82
129115	Intron- Exon Junction	11	322	tgtgtccccttttcacctgg	54	83
129116	Intron	11	445	attaccaatggagcatggca	43	84
129117	Intron	11	810	caacatggccaaaccccatg	55	85
129112	Intron	12	1766	gcggcagggtctccagggtctc	43	86
129110	Intron	12	4813	ttcccttcggagagtagaga	44	87
129113	Intron	12	5848	tgcttgggaaatgcctgcaa	34	88
129114	Intron	12	6936	agctggatgccagaaaggcc	40	89
129111	5'UTR	13	39	tggaagtagtggaagccagg	11	90

As shown in Table 1, SEQ ID NOs 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 60, 61, 62, 63, 64, 65, 66, 67, 69, 70, 71, 72, 73, 74, 76, 78, 79, 80, 82, 83, 84, 85, 86, 87, 88 and 89 demonstrated at least 30%

inhibition of human clusterin expression in this assay and are therefore preferred. The target sites to which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred sites for
5 targeting by compounds of the present invention.

Example 16

Western blot analysis of clusterin protein levels

Western blot analysis (immunoblot analysis) is carried
10 out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western
15 blotting. Appropriate primary antibody directed to clusterin is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

What is claimed is:

- 5 1. A compound 8 to 50 nucleobases in length which is targeted to the 3' UTR, an intron, an intron-exon junction, or nucleobases 106-1402 of the coding region of a nucleic acid molecule encoding clusterin, wherein said compound specifically hybridizes with and inhibits the
10 expression of clusterin.
2. The compound of claim 1 which is an antisense oligonucleotide.
3. The compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO: 18,
15 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 60, 61, 62, 63, 64, 65, 66, 67, 69, 70, 71, 72, 73, 74, 76, 78, 79, 80, 82, 83, 84, 85, 86, 87, 88 or 89.
- 20 4. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.
5. The compound of claim 4 wherein the modified internucleoside linkage is a phosphorothioate linkage.
- 25 6. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified sugar moiety.
7. The compound of claim 6 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.
- 30 8. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified nucleobase.
9. The compound of claim 8 wherein the modified nucleobase is a 5-methylcytosine.
10. The compound of claim 2 wherein the antisense
35 oligonucleotide is a chimeric oligonucleotide.

11. A compound 8 to 50 nucleobases in length which specifically hybridizes with at least an 8-nucleobase portion of an active site on a nucleic acid molecule encoding clusterin.

5 12. A composition comprising the compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

13. The composition of claim 12 further comprising a colloidal dispersion system.

10 14. The composition of claim 12 wherein the compound is an antisense oligonucleotide.

15 15. A method of inhibiting the expression of clusterin in cells or tissues comprising contacting said cells or tissues with the compound of claim 1 so that expression of clusterin is inhibited.

16 16. A method of treating an animal having a disease or condition associated with clusterin comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1 so that expression of clusterin is inhibited.

20 17. The method of claim 16 wherein the disease or condition is a hypercholesterolemia.

18. The method of claim 16 wherein the disease or condition is a cardiovascular disorder.

25 19. The method of claim 16 wherein the disease or condition is a hyperproliferative disorder.

20. The method of claim 16 wherein the disease or condition is a hyperlipidemic disorder.

SEQUENCE LISTING

<110> Isis Pharmaceuticals, Inc.
 Brett P. Monia
 Susan M. Freier

<120> ANTISENSE MODULATION OF CLUSTERIN EXPRESSION

<130> RTSP-0177

<150> 09/659,791

<151> 2000-09-11

<160> 90

<210> 1

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 1

tccgtcatcg ctccctcaggg

20

<210> 2

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 2

atgcattctg cccccaagga

20

<210> 3

<211> 1648

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (53)...(1402)

<400> 3

cgcggaacagg gtgccgctga ccgaggcgtg caaagactcc agaattggag gc atg atg
 Met Met
 1

58

aag act ctg ctg ctg ttt gtg ggg ctg ctg ctg acc tgg gag agt ggg

106

Lys	Thr	Leu	Leu	Leu	Phe	Val	Gly	Leu	Leu	Leu	Thr	Trp	Glu	Ser	Gly	
		5					10					15				
cag	gtc	ctg	ggg	gac	cag	acg	gtc	tca	gac	aat	gag	ctc	cag	gaa	atg	154
Gln	Val	Leu	Gly	Asp	Gln	Thr	Val	Ser	Asp	Asn	Glu	Leu	Gln	Glu	Met	
	20					25					30					
tcc	aat	cag	gga	agt	aag	tac	gtc	aat	aag	gaa	att	caa	aat	gct	gtc	202
Ser	Asn	Gln	Gly	Ser	Lys	Tyr	Val	Asn	Lys	Glu	Ile	Gln	Asn	Ala	Val	
	35				40					45					50	
aac	ggg	gtg	aaa	cag	ata	aag	act	ctc	ata	gaa	aaa	aca	aac	gaa	gag	250
Asn	Gly	Val	Lys	Gln	Ile	Lys	Thr	Leu	Ile	Glu	Lys	Thr	Asn	Glu	Glu	
				55				60						65		
cgc	aag	aca	ctg	ctc	agc	aac	cta	gaa	gaa	gcc	aag	aag	aag	aaa	gag	298
Arg	Lys	Thr	Leu	Leu	Ser	Asn	Leu	Glu	Glu	Ala	Lys	Lys	Lys	Lys	Glu	
			70					75					80			
gat	gcc	cta	aat	gag	acc	agg	gaa	tca	gag	aca	aag	ctg	aag	gag	ctc	346
Asp	Ala	Leu	Asn	Glu	Thr	Arg	Glu	Ser	Glu	Thr	Lys	Leu	Lys	Glu	Leu	
		85					90					95				
cca	gga	gtg	tgc	aat	gag	acc	atg	atg	gcc	ctc	tgg	gaa	gag	tgt	aag	394
Pro	Gly	Val	Cys	Asn	Glu	Thr	Met	Met	Ala	Leu	Trp	Glu	Glu	Cys	Lys	
	100					105					110					
ccc	tgc	ctg	aaa	cag	acc	tgc	atg	aag	ttc	tac	gca	cgc	gtc	tgc	aga	442
Pro	Cys	Leu	Lys	Gln	Thr	Cys	Met	Lys	Phe	Tyr	Ala	Arg	Val	Cys	Arg	
	115				120					125					130	
agt	ggc	tca	ggc	ctg	gtt	ggc	cgc	cag	ctt	gag	gag	ttc	ctg	aac	cag	490
Ser	Gly	Ser	Gly	Leu	Val	Gly	Arg	Gln	Leu	Glu	Glu	Phe	Leu	Asn	Gln	
				135				140						145		
agc	tcg	ccc	ttc	tac	ttc	tgg	atg	aat	ggc	gac	cgc	atc	gac	tcc	ctg	538
Ser	Ser	Pro	Phe	Tyr	Phe	Trp	Met	Asn	Gly	Asp	Arg	Ile	Asp	Ser	Leu	
			150					155					160			
ctg	gag	aac	gac	cgg	cag	cag	acg	cac	atg	ctg	gat	gtc	atg	cag	gac	586
Leu	Glu	Asn	Asp	Arg	Gln	Gln	Thr	His	Met	Leu	Asp	Val	Met	Gln	Asp	
		165					170					175				
cac	ttc	agc	cgc	gcg	tcc	agc	atc	ata	gac	gag	ctc	ttc	cag	gac	agg	634
His	Phe	Ser	Arg	Ala	Ser	Ser	Ile	Ile	Asp	Glu	Leu	Phe	Gln	Asp	Arg	
	180					185					190					
ttc	ttc	acc	cgg	gag	ccc	cag	gat	acc	tac	cac	tac	ctg	ccc	ttc	agc	682
Phe	Phe	Thr	Arg	Glu	Pro	Gln	Asp	Thr	Tyr	His	Tyr	Leu	Pro	Phe	Ser	
	195				200					205					210	
ctg	ccc	cac	cgg	agg	cct	cac	ttc	ttc	ttt	ccc	aag	tcc	cgc	atc	gtc	730
Leu	Pro	His	Arg	Arg	Pro	His	Phe	Phe	Phe	Pro	Lys	Ser	Arg	Ile	Val	
				215				220						225		
cgc	agc	ttg	atg	ccc	ttc	tct	ccg	tac	gag	ccc	ctg	aac	ttc	cac	gcc	778

Arg	Ser	Leu	Met	Pro	Phe	Ser	Pro	Tyr	Glu	Pro	Leu	Asn	Phe	His	Ala	
			230					235					240			
atg	ttc	cag	ccc	ttc	ctt	gag	atg	ata	cac	gag	gct	cag	cag	gcc	atg	826
Met	Phe	Gln	Pro	Phe	Leu	Glu	Met	Ile	His	Glu	Ala	Gln	Gln	Ala	Met	
		245					250					255				
gac	atc	cac	ttc	cac	agc	ccg	gcc	ttc	cag	cac	ccg	cca	aca	gaa	ttc	874
Asp	Ile	His	Phe	His	Ser	Pro	Ala	Phe	Gln	His	Pro	Pro	Thr	Glu	Phe	
	260					265					270					
ata	cga	gaa	ggc	gac	gat	gac	cgg	act	gtg	tgc	cgg	gag	atc	cgc	cac	922
Ile	Arg	Glu	Gly	Asp	Asp	Asp	Arg	Thr	Val	Cys	Arg	Glu	Ile	Arg	His	
275				280					285						290	
aac	tcc	acg	ggc	tgc	ctg	cgg	atg	aag	gac	cag	tgt	gac	aag	tgc	cgg	970
Asn	Ser	Thr	Gly	Cys	Leu	Arg	Met	Lys	Asp	Gln	Cys	Asp	Lys	Cys	Arg	
				295				300					305			
gag	atc	ttg	tct	gtg	gac	tgt	tcc	acc	aac	aac	ccc	tcc	cag	gct	aag	1018
Glu	Ile	Leu	Ser	Val	Asp	Cys	Ser	Thr	Asn	Asn	Pro	Ser	Gln	Ala	Lys	
			310					315					320			
ctg	cgg	cgg	gag	ctc	gac	gaa	tcc	ctc	cag	gtc	gct	gag	agg	ttg	acc	1066
Leu	Arg	Arg	Glu	Leu	Asp	Glu	Ser	Leu	Gln	Val	Ala	Glu	Arg	Leu	Thr	
		325					330					335				
agg	aaa	tac	aac	gag	ctg	cta	aag	tcc	tac	cag	tgg	aag	atg	ctc	aac	1114
Arg	Lys	Tyr	Asn	Glu	Leu	Leu	Lys	Ser	Tyr	Gln	Trp	Lys	Met	Leu	Asn	
	340					345					350					
acc	tcc	tcc	ttg	ctg	gag	cag	ctg	aac	gag	cag	ttt	aac	tgg	gtg	tcc	1162
Thr	Ser	Ser	Leu	Leu	Glu	Gln	Leu	Asn	Glu	Gln	Phe	Asn	Trp	Val	Ser	
355					360					365					370	
cgg	ctg	gca	aac	ctc	acg	caa	ggc	gaa	gac	cag	tac	tat	ctg	cgg	gtc	1210
Arg	Leu	Ala	Asn	Leu	Thr	Gln	Gly	Glu	Asp	Gln	Tyr	Tyr	Leu	Arg	Val	
				375					380					385		
acc	acg	gtg	gct	tcc	cac	act	tct	gac	tcg	gac	gtt	cct	tcc	ggt	gtc	1258
Thr	Thr	Val	Ala	Ser	His	Thr	Ser	Asp	Ser	Asp	Val	Pro	Ser	Gly	Val	
			390					395					400			
act	gag	gtg	gtc	gtg	aag	ctc	ttt	gac	tct	gat	ccc	atc	act	gtg	acg	1306
Thr	Glu	Val	Val	Val	Lys	Leu	Phe	Asp	Ser	Asp	Pro	Ile	Thr	Val	Thr	
		405					410					415				
gtc	cct	gta	gaa	gtc	tcc	agg	aag	aac	cct	aaa	ttt	atg	gag	acc	gtg	1354
Val	Pro	Val	Glu	Val	Ser	Arg	Lys	Asn	Pro	Lys	Phe	Met	Glu	Thr	Val	
	420					425					430					
gcg	gag	aaa	gcg	ctg	cag	gaa	tac	cgc	aaa	aag	cac	cgg	gag	gag	tga	1402
Ala	Glu	Lys	Ala	Leu	Gln	Glu	Tyr	Arg	Lys	Lys	His	Arg	Glu	Glu		
435					440					445						
gatgtggatg	ttgcttttgc	accttacggg	ggcatcttga	gtccagctcc	ccccaaagatg											1462

agctgcagcc cccagagag agctctgcac gtcaccaagt aaccaggccc cagcctccag 1522
gcccccaact ccgcccagcc tctccccgct ctggatcctg cactctaaca ctcgactctg 1582
ctgctcatgg gaagaacaga attgctcctg catgcaacta attcaataaa actgtcttgt 1642
gagctg 1648

<210> 4
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Primer

<400> 4
tccgtacgag cccctgaa 18

<210> 5
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Primer

<400> 5
tgagcctcgt gtatcatctc aag 23

<210> 6
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Probe

<400> 6
tccacgccat gttccagccc t 21

<210> 7
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Primer

<400> 7
caacggattt ggctgtattg g 21

<210> 8
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Primer

<400> 8
ggcaacaata tccactttac cagagt

26

<210> 9
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Probe

<400> 9
cgcctgggtca ccagggtgc t

21

<210> 10
<211> 8133
<212> DNA
<213> Homo sapiens

<400> 10
gccatgttgc ccaggctggt ctcaaactcc taagctcaag taatcctcct accttggcct 60
cccaaattgt tgggattata gatgtgtgcc actatgccca gccaatgtaa gattttgtag 120
tatattagtg ttgctcctgt cctctgctgc agggcttttt tgattgggac tcagtgaatt 180
gctccaatcc ctgaagtcac atcagttggc ccttagccga gcgggggtgg atatcattgg 240
tggccaaaga tgacagtga tgaacctgaa atgttgggcc ttgtgacttt tgggcctcca 300
ggtgtctcaa aactgtcccc catggaggga gataaaagga aagagcatgg acctgacaga 360
tgggggtgctg ggggctggtc ccagctgggc tggttggtcac ttgctgtgtg actgttacag 420
ccatgggcag ggcctggcct ggctcaccag ggggtgggag gccaggaggc cgtggccttg 480
gtgagcttct cctaactgtg cccatgctgg ctgtcccagc ttgaggagtt cctgaaccag 540
agctcgccct tctacttctg gatgaatggt gaccgcatcg actccctgct ggagaacgac 600
cggcagcaga cgcacatgct ggatgtcatg caggaccact tcagccgcgc gtccagcatc 660
atagacgagc tcttccagga caggttcttc acccgggagc cccaggatac ctaccactac 720

ctgcccttca	gcctgccccca	cgggaggcct	cacttcttct	ttcccaagtc	cgcctcgtc	780
cgcagcttga	tgcccttctc	tccgtacgag	cccctgaact	tccacgccat	gttccagccc	840
ttccttgaga	tgatacacga	ggctcagcag	gccatggaca	tccacttcca	cagcccggcc	900
ttccagcacc	cgccaacaga	attcatacga	ggtgagaagg	ggtggaagct	catggccttt	960
tgagcaactc	gtagatgct	gagaaccatg	ccgagggctc	agcgggtgtc	atctcgattt	1020
ttctccagca	atatcacaag	ggtgatatta	tccttattta	aagaggaaaa	aaactgagct	1080
gggcatgggtg	gctcatgcct	gtgatgccag	cactttgaga	ggccaaggcg	ggaggatcat	1140
ttgaggccag	gagtttgaga	ccagcctggc	caagatagtg	agaccctgtc	tctacaaaaa	1200
taaaaactta	aaaaattagc	cgggtgtggt	ggtgcacacc	tgtagtctca	gctactcggg	1260
aggctgaggc	aagagagtca	cctgagcctg	gaagttggag	gctgcagtga	gctatgattg	1320
caccattgca	ttccagcctg	ggcaacagag	tgagaccctg	tctctaaatt	aaaaaataaa	1380
taaaaataac	aataggaatc	agtggagtcc	atctctgcat	ggctggatga	ctgactcttc	1440
ttccctcgtg	tgtccccaga	aggcgacgat	gaccggactg	tgtgccggga	gatccgccac	1500
aactccacgg	gctgcctgcg	gatgaaggac	cagtgtgaca	agtgccggga	gatcttgtct	1560
gtgggtgagt	cgggggtccag	accacaagcc	gtccccctg	atcccttgtg	tcctgggggtc	1620
actggggcct	cactggtgct	gcctttatgg	agtcagacag	ataagcgttt	ggattccagc	1680
tctgcagcct	ttgagctgtg	tcccggggca	ggtcctgagc	ctcatgcagc	ttcggttcct	1740
catcttagaa	tgagatgatg	atgcgaggct	gtccctgaag	tcgggtgagat	gtcgtagag	1800
atgcaaaagt	gcctccacc	tggtcggccc	catgttgaaa	aaagcttggt	gaaaaaagtc	1860
atccccctgg	gactccccgg	tgattctggt	ccaagcgcc	aagcagtagg	catcttcatt	1920
ttcctctgca	gattatgaca	ttgcagacag	tatgtgtttt	gtttaacaaa	actgaccaga	1980
ggccaggcac	tgttctaaac	actcgacata	catttcctca	tttctcaga	atgaccctct	2040
gaggaaactg	agccacagaa	aggттаатаа	cttatccaag	attgaccccg	acatgggcga	2100
gctgggcttc	aatcctaggg	cgctgtgttc	tctcctgggg	cccctcgcag	cctctgccac	2160
agaagtcacg	ggtctcagta	cctgggcatc	caagcaatag	tccctttggg	cggttgggtg	2220
gtcccctagg	caaagggaat	atttcccttt	aactgtcccc	ctccgtttca	ccagctctgg	2280
ttatgggtta	acttctttcc	acttagagat	aacagctgtg	acagtatttg	gactagttcc	2340
tggtacacag	cagttcatac	tcacaaagag	ttaattgttt	ccccttggtc	aacagcttat	2400

cgatctggtg gctttgctct tacttaatgc ttagtttgag tttgccatgg caggccgcca 2460
 gggctctagtt aaacattcct agcctcactc ctataatttt agaagccact gcaaaataaa 2520
 cagttgtgct ttaacaggct gaagtataag ttgctgtaga tgagtgcaca accaggcctt 2580
 ggggcttttt ctataaaaaa tatcatagag tggcatcaat tacatgggtac ctcaccacaa 2640
 gaaagtcatg ttaggggtctg agaaaagatg tcagatgcct gtgcccagat tggacctctt 2700
 atagctgatt tttactctgt tgcccaggct gggtcaggctc tggcccaatc ttaacagtca 2760
 ttgattacag ttgagagtgc agccagcgcc agtcttatca gtcattgatt atagctggcg 2820
 tacagtggct ctatctcggc tcaactgcgac ctccgcctcc tgggttcaag tgattctcct 2880
 gcctcagcct cccaagtagc tgggagtgcg ggtgtgcacc accacacca gctaattttt 2940
 gtatttttag tagagacagc atttcactat gttggccagg ctggtcttga actcctgacc 3000
 tcaagtaatc tccccgcctc ggccctccaa agttctggga ttacagggtg gagccactgt 3060
 gcctgacctg agatagattc ttagagaatt attggtaaga ataattctct aagctgagct 3120
 aaatagtcta cactgaagag gactgcctac tgttatttaa ggtgcttgca accatataag 3180
 catgtactgc ctgggaactc tagatgagga tttctcaatt tcagcgctgt tgattttttt 3240
 tttttttttt gagacagggg ctctctctat caccagcct ggagtgcagt ggcaccatta 3300
 cagctcactg cagcctagac ctcttgggct gaagtcatcc tcctgcctca gcctcctgag 3360
 taacagacta cagggtgtgct ccaccatgct tggctaattt ttttattttt agtagagatg 3420
 gggctcttgct acattgcccc agctgggtct taactcctgg gctcaagtga tcctcctacc 3480
 tcagcctccc agagtgcctg gattacaggt gtgagcagtg ctgacatttt ggaccaggctc 3540
 attctttgtc gttgggggct gtcctgagca gttcaggggtg tttggcagca ttcttggcct 3600
 ctgcccacta gaggtcagca gctcccttcc ctttggttggtg acaaccagct tcagaacttg 3660
 ctaaattctcc ctgggtgaca gcgtccacag tagagaacct ctattctaga ctaagcctca 3720
 gctcttaagg atttttctta ttttattatt atttttttta gacaggggtct cgctctatca 3780
 cccaggctgg agcgtagtgg cgcaatcttt gctcactgca acctctgctt cctggggttca 3840
 agcgatttct cctgccccag cctcctgagt agctgggatt acaggcgtgc accgccacgc 3900
 ctggctaatt tttatatttt tagtagagac agggtttcac catattggcc aggctgggtct 3960
 caaactcttg acctcaagtg atcagcctgc ctcagcctcc caaagtgctg ggattacagg 4020
 tgtgagccag cacgcctggc tagtttttct tattttttaa tttttttttg gtaaaataat 4080

gatgtttatt	tattacatat	ttattttcaa	actggcatct	tgtagtaat	tctgtttctt	4140
tccccaccta	acattttgtt	tactataaat	gatttcagtc	atcatcctaa	agcatatgca	4200
aatctccct	tcccctgact	cacgtttgat	gtacctgcct	ctggatattt	ttgaaatacc	4260
ttagggggag	aaaaacagta	gttttaagag	ctagtggaca	gtttccaggt	cttaatgaat	4320
ctgacaacct	gcagcccagg	gccaagagga	atgaattctc	ttttccctgc	tctcttgatg	4380
aactcactga	ccagccatgg	gcggcaggtg	ggcaggcaag	gaccctggc	caccaggtgc	4440
cagtgcac	gctgcataa	ctcctggcac	cagaactgcc	acctctacag	acatgctcaa	4500
aagacaagtt	tggaccgggt	gcattggctc	acacctgtaa	tcccagcacc	ttgagaggcc	4560
gaggtgggtg	gaccctgag	gtcaggagtt	tgagaccagc	ctagccaaca	tggtgaaacc	4620
ctgtctctcc	taaaaataca	aaaaaatcac	ccgggggtgg	tggcaggcac	ctgtaatccc	4680
aactactctg	gaggctgagg	caggagaatt	gcttgaaccc	gggaggtgga	ggttgcagtg	4740
agctgagctc	gcgccattgc	actccagcct	gggaaacaag	agcgaaattc	tgtctcaaaa	4800
aaaagacaag	cttggaggat	tgtccagaac	cacagatcca	gggtaggaaa	agcccaagct	4860
taggagctga	agaccctgg	tcaatcccgg	gccagagat	catttattct	atggcttttag	4920
gtaagctatt	tattgatact	tctgtgggcc	tcagtttcat	tattggtaaa	aattatttca	4980
ttattggtaa	aattaggact	taagtcctaa	tccttaagtc	agaacagatc	caattcttag	5040
agaaaaagga	tatccagaga	gaactttctg	cgggtgtctg	gacgcaggca	gtgccacacg	5100
aatggcagct	gtgagtaata	ttcctcctct	ctggaaatga	ttcccgggag	gactagggca	5160
acgagagcca	ctccaggtct	gagaacatgg	agaacttgag	atcagtgcct	ttggaagtgt	5220
ggtcaacaca	gtttgtcacc	aaagagataa	gggtctggca	cccaaagata	aatgaatgat	5280
gttacgaagc	acactgttta	ggtcagttgg	cgtatttttc	cagagcaagg	cttctcaggc	5340
tgggcgtgg	ggctcacacc	agtaatccca	gcactttttg	ggcagatggg	ttgagcccag	5400
gagttcgaga	ccagcctgga	caacacagag	aaaccccg	tctacaaaaa	atacaaaaat	5460
tagctgggca	tggtagcatg	tgcttatagt	cccagctact	caggaggctg	aggttggagg	5520
acagcctgag	cctgggaagt	caaggctgca	gtgagccgag	atctcaccac	tgtattccag	5580
cctaggcaac	agagcaaaac	tctgtctcaa	aaaaacaaaa	acaaaaacaa	aaaacccaaa	5640
agactttctg	gatgacggaa	gcagtgtcta	gattcacatt	ctgaggcaaa	acctttattt	5700
tgtcgtggac	aattccagtt	tgtggccctt	cccttaggga	agcactgcct	ttgttcccgc	5760

tgcattgtgct aacttccatt cattcatggg tctatccctt tgtagccttc ccttcacact 5820
tctcacttgc gtttcttcca tctctgggca gactgttcca ccaacaaccc ctcccaggct 5880
aagctgcggc gggagctcga cgaatccctc caggctcgctg agagggttgac caggaaatac 5940
aacgagctgc taaagtccta ccagtggag atgctcaaca cctcctcctt gctggagcag 6000
ctgaacgagc agtttaactg ggtgtcccgg ctggcaaacc tcacgcaagg cgaagaccag 6060
tactatctgc gggtcaccac ggtgagctgt gtcccggcca catgctgtgg ctcgaggacc 6120
gagctgtgat cgggagcagg ggcattgtgt cttttgactg agcatttata acacggcaga 6180
aaatagaaaa ctttaggcgc ccctgttgcc ttgaagcctc atcaccact cagggaataa 6240
ataaccctgc ttacaaagg agcaaagtaa gagagggtcc acagcttggc caagggtgtga 6300
tagctgacag atgacttggc cgggtatttg aacctgactg cctggctgcc aagcctgtat 6360
tttggtgttg ttgtttttgt ttggtgcac aaatctgtga ataaaccaga agcctctgtt 6420
cttttctcaa agctacaagg ctgccctctg gcatgtaaaa tggcttatga attagtacat 6480
cactctctgc cagtataaaa aacttctctc taggcagac atggtggctc atgcctgtaa 6540
tcccagcact ttgggaggca gaggcaagag gattgcttga ggccaggaat ttgagaccag 6600
cctgggcaac acagcaagat tccctctcta caaaaaatac aaaaatcagt cagggtgtgg 6660
ggcacacact tgtagtccca gctattcagg aggctgaggt gggaggattg cctgagccct 6720
gaagtggagg ctgcagtgag ctgtgatcac gccactgcac tccagcctgg gtgacagagt 6780
gagactctgt ctcttaaaaa atatatatat ataaaataat aaaataaagt taaaaaatca 6840
aataaaactt atttctagta ctgggaactc ttctttttct tttctttctt cctccaggc 6900
cctctggatt ctttttctac cctactctga ccaagggctg cctaaagcaa atgtttggaa 6960
accactttta ttctttgggg tgctccctgg ctggtcattt gcagatgaca ttgccccaa 7020
cacatgagtg tctgtgaacc aggtccgttc tgtccactga gctgtactta cgtctagatg 7080
tataagaagc atgggggtcag ctctctaggt tccttgagg agcaggagga cttccttata 7140
agaagcctga cttctgttgc agagcgcagt cattttgacc acagtgtttc agctcttccc 7200
ttttctcttg ttccatttag gtggcttccc acacttctga ctcgagcgtt ccttccgggtg 7260
tcactgaggt ggtcgtgaag ctctttgact ctgatcccat cactgtgacg gtcctgtag 7320
aagtctccag gaagaaccct aaatttatgg agaccgtggc ggagaaagcg ctgcaggaat 7380
accgcaaaaa gcaccggtaa gcaggcgggc ctttcctgcg gcctgcaggg ccagtgagt 7440

```

ctctgggagc cacaaaaaaa caaaciaaagt gcagactcta tagcctgggtg ggaacgactc 7500
cgcccggagc cagagcccaa gaaciaaagcc aggaagttac gggggaattt tatttttcct 7560
ttggaggatg ttttactttg gaggataact gttttttatt tcaggaggga gtgagatgtg 7620
gatgttgctt ttgcacctac gggggcatct gagtccagct cccccaaga tgagctgcag 7680
ccccccagag agagctctgc acgtcaccaa gtaaaccagg cccagcctc caggcccca 7740
actccgcca gcctctcccc gctctggatc ctgcactcta aactcgcact ctgctgctca 7800
tggaagaac agaattgctc ctgcatgcaa ctaattcaat aaaactgtct tgtgagctga 7860
tcgcttggag ggtcctcttt ttatgttgag ttgctgcttc ccggcatgcc ttcattttgc 7920
tatggggggc aggcaggggg gatggaaaat aagtagaaac aaaaagcag tggctaagat 7980
ggtataggga ctgtcatacc agtgaagaat aaaaggggtga agaataaaag ggatatgatg 8040
acaaggttga tccacttcaa gaattgcttg ctttcaggaa gagagatgtg tttcaacaag 8100
ccaactaaaa tatattgctg caaatggaag ctt 8133

```

<210> 11

<211> 940

<212> DNA

<213> Homo sapiens

<400> 11

```

aagcttgaac tggagcaagg gtaggcactt gcatgctggg tggccagcct atgggaaggc 60
tcgccctggg gcagagggcc tggcaccag cagctctttg agtgcagag cctgtggtct 120
ctgtgtgctc agccagcctt gtgtcttcct gtaggatgcc ctaaatagaga ccagggaatc 180
agagacaaag ctgaaggagc tcccaggagt gtgcaatgag accatgatgg ccctctggga 240
agagtgtgag ccctgcctga aacagacctg catgaagttc tacgcacgcg tctgcagaag 300
tggctcaggc ctggttggcc gccaggtgaa aaggggacac atgagtggcc aaggctctga 360
gtggggaagg aggggagcct agtgaaatat gcttcattcc gcatgccaga tgcaattgat 420
tagcattggc tggcttgccc agagtgccat gctccattgg taatgtctgg catgagtaga 480
gagagtggag tcatcaaaag gatgtaggcc aggtatctgc cttctcttag aaaactcatg 540
cagcagtgtc tagctggatg acataataaa ctgcttcgtg ggatgcagag ccctgtgtca 600
cttatgtgga aggatttaag aatTTTTTTT tttttttgag acagggtctc actctgtcac 660
ccaggctgga gtacagtgat gtgatcatgt ttcactgcag cttcgacctc ctgggttcag 720

```

gtgatcctcc cacctcagcc tcccaagtag ctgggactac aggcacgtac caccacaccc 780
 agctaatttt tgtatTTTTT ttttgtaaAC atgggggtttg gccatgttgc ccaggctggt 840
 ctcaaactcc taagctcaag taatcctcct accttggcct cccaaattgt tgggattata 900
 gatgtgtgcc actagtccca gccaatgtaa gattttgtag 940

<210> 12
 <211> 7610
 <212> DNA
 <213> Homo sapiens

<220>
 <221> unsure
 <222> 5461
 <223> unknown

<220>
 <221> unsure
 <222> 5462
 <223> unknown

<400> 12
 gacctgcagg tcaacggatc cattcccgat tcctcatcgt ccagatggaa gaaactgagg 60
 cccaagggca aagtgattag tccgaggtca ccagtggtct aggggcacac ctaggactgt 120
 aatcagactt tcatggacct ggtctggggt ctcccactta gtcatgggcc ttgaagattc 180
 cccgaggctg cctcctgaaa aggactgggg tctagtggcc cctggacgtt gggcaagcaa 240
 gggactgggc ctccatgttg tgctccata gtctgatcc tgaactggaa aactcagccc 300
 ctgaccacgc agctctcctt taagcccctt tgtttcacat ggttttcaaa gtctgccacc 360
 cacagtgggg ctgcctgtac ccgccctgtc caccattgc ccagctgtc agccccttga 420
 cttctctcct ggggcttaaa catccctggc tccaaaatgg gcagctcact ttcttcccca 480
 agaagtagct gcacctccag ggttcctaga tttgccctc cttgccaggg ggaggggtgg 540
 ctgcgacagg agattctccc tgctctcagc agaaggaact ccagcagttg gagaccagca 600
 aaccctctg gacacagatc tgatttccta actgggaagg ctcagggcaa aataaaaatt 660
 caggtccact gggtcaaaaa ctatgaagaa tttcaagacc gtcacagtag cccattaaac 720
 caaacgtgga tctgcaaggg tcccacagcc atgaagccca ccctgcttgg ttgggttcca 780
 aaaagatggg gacagtgatt gcttaagctc tgtggatcaa ggaccccgga gaggccttct 840
 ggctctccac atatctgctc tgatcactcc taaacacaat tctgtttcct ccaggcctgg 900

```

cgggtcagtc cagggacccc catcagtgtg atgtttccag gagtaggcgt ttcaatactt 960
cctgtgctct cttctccagc acaaggcccc tctccatccc accctcatta tgtctgactc 1020
tttactatth aaatgggtca agagaagtgg cgcttgtgta atgtgaaggt taaggtcagt 1080
agggccaggg aactgtgaga ttgtgtcttg gactgggaca gacagccggg ctaaccgcgt 1140
gagagggctc ccagatggca cgcgagttca ggctcttccc tactggaagc gccagcgccg 1200
cacctcaggg tctctcctgg agccagcaca gctattcgtg gtgatgatgc gccccccgc 1260
gccccagccc ggtgctgcac cggccccccac ctcccggctt ccagaaagct ccccttgctt 1320
tccgcggcat tctttgggcg tgagtcatgc aggtttgcag ccagcccca aggtgtgtgc 1380
gcgaacggag cgctataaat acggcgccctc ccagtgccca caacgcggcg tcgccaggag 1440
cagcagcatg ggcacagggt ccgtgaccgg tgagatgtcc ccgtcttccc tacccttgag 1500
cagagccaca ccaggacgga tgggcgggca ggggatggca gccaggcaga gagggatgac 1560
acagctcgca gtcacaaccc ctgcgctttc gacggagccc aggaagccag ggaggggagg 1620
tgccggagcc ccatcaccag gcagctgagc cagggggccgc gcaaccgccg cctgatgagc 1680
acgagcttca cgcaaccaca attctgtggt gggggggtaa atagaacaga tataatgatc 1740
atcctttcgc aaagatgggg aaactgagac ctggagacct gccgcgttgg cagaccag 1800
ctagcaggtg acagagctgg cctgcaccga gctccttctt gcagcatatc ctctgcgaag 1860
atgcggatct ctcagttgtg gctttcggct tgcattcatg agtcattctag ttttcttcta 1920
aattctctag ctctctggac actgttgccct gtaagtatga ggctgcggat ttcagtatat 1980
ctgcaaccac cgaaatccga ctttttctgc ctctaattgc atctgaggtg catcagagaa 2040
aagtcacaca agatccacca ggcctcagac ctctgattcc acagtctcat ttacagatg 2100
ataatctgag gcctggagag gtttaggact ggtgcccaaca ctaaacagca aataagtatc 2160
agaattggga ttcgagccaa agcctcttga ccttccagaa tttctggacc tagttaaaaa 2220
aaatatgatt tttattatta ttttttaaac ggagagggtta ggaatttaaa ggaaagtaca 2280
gatactatat aaaaaaagat gcccatgaaa atgttaagtt ataataatag tggagcattg 2340
ggcacaactg aaatggccaa tcttgtgaga atggtaaaat aaacttaggt ccgtgagtaa 2400
gtggagtatt acatagccat aaaagtatgc ccttaaagaa tatttgaaga tggatgaatg 2460
gaagaatctt gtataaactg catggaagac agaaggaaat ataccacagt gctaaccctt 2520
gcctctgggt gatatgaatt accggtgatt atttttctta ttttcctttt ggtttagttt 2580

```


tctccatttg aagaagcaga taggagccgg ggctttggga ttgaaaccct caccatctgt 2640
gtgccctctt cactgtcttc ccatacctccc cacggctccc tggtcacagt cattgatttt 2700
ctttctttct tttctctttt tttttttttt tcttgagacc aagtctcact ctgttgccca 2760
ggctggagta gagtagcgcc atctcggctc actgcaacct ccgccatccg gggtcaagca 2820
gttctcatgc ctcagcctct gagtagctgg gactacagcc gcatgctgct acatccggct 2880
aatttttgta tttttagtag agacatgggt tcaccacctt ggccaggctg gtctcgaact 2940
cctgatctca agtaatccag cctgtcttgg cctcccaaag tgctgggggtg acagggtgtga 3000
atcaatgcgt ccctgccagg tcattgattt tcttaagcct ctagccctgc cctgcttgga 3060
aacgttttgg gaagctgctc agttcaaagt tcccaggagg gtgtgcctgg aggggagttg 3120
ctcccaaagt ctgcctgctc ccccgcccc ccctgcccc cccccccgc catcttctcc 3180
tctctctctt ccctgagca gccctttgt ccacagaacc ggcttttct ggtagaagga 3240
gcaaggccaa gtggtttaag ccttcttagg gagaatgagg ctgtgtggta gtgctgggga 3300
ctcgagggcc ttgcgttggc atggctcttc caccagggc agctggcagc caggctccca 3360
ggaggcagag gagatgaggg gggagggtgag tccgagcaaa ggaaaggagg tcggctgtgc 3420
agtcacgggt ctagaacatt cattggatca gcagcatcca tatcacctgc agactggctg 3480
gaaaagcagt ctcagaacca acattataac cagccctgca gtgattcata agtactttaa 3540
aaagtgggtca atcatttcag caaagcagag ccacacagtc cgggggacca cagggtggcct 3600
ctgtgtgctt gtctcggttt tcctgcccct ctccagacat gttgattaga cactgccaat 3660
gccagcctc agacctcagt ctaatttgga agtagtcaga atttactatg attacataag 3720
accctcgtgt ttacagaaca cattcccctc tctgaggtct ggattagatc cattttacag 3780
atgaagaaac tgaggctcag atatttaagt gacttggaat caaggaaaga atactggaca 3840
tggggctggg agggctgggc tctcatccca gggttaccat gagcatgctg tggactctag 3900
ggagtccatg ccctctctgg cgttcagctc accgctaggt agagagggtg ggtgagagaa 3960
cgacctcctt cccaggctctg agctggatgg ttcaccaggg accccaggct ccctggacga 4020
gactctgtgc ccgctgctga gtctggaatt cctttctgt atcttgccct tgcgtgcccc 4080
attcttcatg gccagcacc ctgtcttctg gtcagaacct agttctgaat gggtttttcc 4140
agaagttgtt gctttcaggg gccctggca gagagggtgt tctggctggc tttgtctctc 4200
tggcatgaca aaggctctgt tcctgctgga ggcatttcag ggctcagtgg gcagctgggg 4260

cagacgctga gaccacagcc ttcttgggtga gcccggtctc cgccccctac cccatctctg 4320
ggaaggcgct gaccccatct cttctccac gctgctccct ggctctttgc gcctgattac 4380
ttctcatgag aggcactcct tgttaatgtg ctactgagtg tccagatggg cctgctgggc 4440
tgagcgggct ttggatgtga accatttcag gaaggggaac ccatcgctct gttgggttctg 4500
tgatggcaaa tgggtgagct cagataacga gttcttggga ggggcatggg gggggtggag 4560
tgcaggggga ggggtttctg ttttattgac aacagcctca gcttctggga aagggtccat 4620
tgtgtaagac cggggctatg gctgtgcccc gtggctcagg gcagccagcc agtgggtggca 4680
ggaacactgg cagggcagcc tcgtgtcggc ttagagggga tgggcagtgt ggagggcctg 4740
gcagagcaag aggactcatc cttccaaagg gactttctct gggaagcctg ctctcgggc 4800
cactgcgaac cctctctact ctccgaagga attgtccttc ctggcttcca ctacttccac 4860
ccctgaatgc acaggcagcc cggcccaagt ctcccactag gatgcagatg gattcggtgt 4920
gaagggctgg ctgctgttgc ctccgcgtct tgaaagtcaa gttcaggtgg tgctgagact 4980
ccctgggggc tgcagcgctg tgggtgaatgg ggagcgtctg ctgggggtgaa ggttttaggtg 5040
cacattgcag aggacgtggc tgggtctctgg gatgcagtc ctctgtggag gtggcatggg 5100
gagggacgga tgcattgacct aagggtggta ttttcagtgt ctgacatgat cgataccact 5160
ctggacaagg aggccaggat gcagaaagcc tgtgtgcctc gctgattgtc ggggaggatg 5220
tggcttggac aagagcctgg ttctctcgat gccaggggtc ttgtttcttc cactcaacat 5280
tgctgtcctg cagtccctcc ctccctgcac ctctgcctt cgctttcatt cgaggtgtcc 5340
atggcaagtc tggtcatttc cccccatttc ctccaggaata aaagttgcag cagtgcctgc 5400
tgtggggaca gctgagggca gtgaggctgg ggagctgctg cagggcgagg tgggcgggac 5460
nnagcaggct gtctagctgt tcccatgatg gtctcctgtt ctctgcagag gcgtgcaaag 5520
actccagaat tggaggcatg atgaagactc tgctgctgtt tgtggggctg ctgctgacct 5580
gggagagtgg gcaggctcctg ggggaccaga cggcttcaga caatgagctc cagggtgagt 5640
agaccaagca tgatgttcct ctggccacag ggtgatgagg tcagagggca gggtagctaa 5700
ttctgctcag tgctctctta tcaggcccca gtgttacaga ccgtttttat cttgtgcact 5760
gggtctgggt gcctgtgtct gggccactc tgagcctcag ctcccaggcc cctgggttcag 5820
gctctgcgtg catcagactg ccggcatttg caggcatttc ccaagcactt tcggctgttg 5880
catttcattc agctcttccc ctcccaggcc ccttagccca gctcccaggc ctctccaca 5940

```

aagctgtgtc tggaccaccg gagctcttat ccctctcccc tttggagtgc ccagagctta 6000
tcctctctgt gagctgacgg tttctgcagg atcattgtta aaaacccaga tcagacatgg 6060
gtgtgagtct gtttcacctc ttctcagctg ggtgactttg ggccactatc ttgatctcat 6120
gacactcccc ccacccccca ttttattgag atataattaa caaataaaaa ttgtgtatat 6180
ttaaggtata tgacgtgatg ttttgaaatg cacatacatt gaaatgatga ccagttttta 6240
tggtgggacg gtgggaagac ttaaaatcta ctttcttagc aaatttccag ttatgatatg 6300
gtgttattaa ctataagcac cacctgtatg ttagacctcc agaacatact cctcctacct 6360
gatgaacact ttgacccttt atcatatcac acttcccatg tctccctctg cgaagtgggc 6420
acggcggggg gctggagcat tacgtaaact gcacatgaag tgtttggcgc agtgcttggc 6480
atgggataaa caccagtga gtagcactta ggtgacacag tgtttcgctg catttgtcac 6540
cagtgtctatc cttactcatt tactcatctt cttattcctg tcgcctggca ctgcattgga 6600
acaaagaaat acacatatct gtttaaactg aactctagaa agatttgtgt ccaaataaac 6660
aatattttat attttgatgc tgcaaactg acacttctgg gttttttttt tttccttgcc 6720
aagtttcttc tgcaccacgc tcattctcca ggggcacatg gcagtggctg ggcataactc 6780
tggtgtgtcc ggctcccatg gtctgcattt ctaagcagta ggggtgcagtc agcaaggagc 6840
ctgtgatggg agcctgtgcc agggcaaggc tggggcatgc tgctgcctgc tggcaggagt 6900
gggggtccca gccttgacag cccctgaact gaacgggcct ttctggcatc cagctcattc 6960
cagggtcctg aggccacctc ttctctcgc ctcattctgc ctcttgact tctcttgacg 7020
aaatgtccaa tcagggaagt aagtacgtca ataaggaaat tcaaaatgct gtcaacgggg 7080
tgaaacagat aaagactctc atagaaaaaa caaacgaaga gcgcaagaca ctgctcagca 7140
acctagaaga agccaagaag aagaaagagg tcaggaggag ccgctaccgc ctccctgcct 7200
tgaccatccc actggagggg agggaggggg tcactgcgcg gtgccctgct ggttgccatg 7260
gtgaccgcga gtcctcccag gctgtgtcag ctgatgctga ggctgcagtt aagaagcagg 7320
gaaggttcat ttgcttctga aagcatcagg gagtgagatc ttggatctgg ttttgttatg 7380
agcctggccc agggctaata ccagattcat ttcaatagat gtttctaage cctgatcacg 7440
tgctagtacc aagcaggctc tgggtggggg ggcggcaggg gccagacagg cgtggcgctc 7500
aaccttcagg aagcttctag gagttaggga acagttggat cttgaaggat gagggggttc 7560
ttaagccag gtgggaaggg gattccaggg gggcgaatga ggggaagctt 7610

```

<400> 13															
ctgcgaaccc tctctactct ccgaagggaa ttgtccttcc tggcttccac tacttccacc 60															
cctgaatgca caggcagccc ggcccaagtc tcccactagg gatgcagatg gattcgggtgt 120															
gaaggggctgg ctgctgttgc ctccggctct tgaaagtcaa gttcagaggc gtgcaaagac 180															
tccagaattg gaggcattg atg aag act ctg ctg ctg ttt gtg ggg ctg ctg 231															
Met Lys Thr Leu Leu Leu Phe Val Gly Leu Leu 10															
ctg acc tgg gag agt ggg cag gtc ctg ggg gac cag acg gtc tca gac 279															
Leu Thr Trp Glu Ser Gly Gln Val Leu Gly Asp Gln Thr Val Ser Asp 15 20 25															
aat gag ctc cag gaa atg tcc aat cag gga agt aag tac gtc aat aag 327															
Asn Glu Leu Gln Glu Met Ser Asn Gln Gly Ser Lys Tyr Val Asn Lys 30 35 40															
gaa att caa aat gct gtc aac ggg gtg aaa cag ata aag act ctc ata 375															
Glu Ile Gln Asn Ala Val Asn Gly Val Lys Gln Ile Lys Thr Leu Ile 45 50 55															
gaa aaa aca aac gaa gag cgc aag aca ctg ctc agc aac cta gaa gaa 423															
Glu Lys Thr Asn Glu Glu Arg Lys Thr Leu Leu Ser Asn Leu Glu Glu 60 65 70 75															
gcc aag aag aag aaa gag gat gcc cta aat gag acc agg gaa tca gag 471															
Ala Lys Lys Lys Lys Glu Asp Ala Leu Asn Glu Thr Arg Glu Ser Glu 80 85 90															
aca aag ctg aag gag ctc cca gga gtg tgc aat gag acc atg atg gcc 519															
Thr Lys Leu Lys Glu Leu Pro Gly Val Cys Asn Glu Thr Met Met Ala 95 100 105															
ctc tgg gaa gag tgt aag ccc tgc ctg aaa cag acc tgc atg aag ttc 567															
Leu Trp Glu Glu Cys Lys Pro Cys Leu Lys Gln Thr Cys Met Lys Phe 110 115 120															
tac gca cgc gtc tgc aga agt ggc tca ggc ctg gtt ggc cgc cag ctt 615															
Tyr Ala Arg Val Cys Arg Ser Gly Ser Gly Leu Val Gly Arg Gln Leu 125 130 135															
gag gag ttc ctg aac cag agc tcg ccc ttc tac ttc tgg atg aat ggt 663															
Glu Glu Phe Leu Asn Gln Ser Ser Pro Phe Tyr Phe Trp Met Asn Gly 140 145 150 155															

gac cgc atc gac tcc ctg ctg gag aac gac cgg cag cag acg cac atg Asp Arg Ile Asp Ser Leu Leu Glu Asn Asp Arg Gln Gln Thr His Met 160 165 170	711
ctg gat gtc atg cag gac cac ttc agc cgc gcg tcc agc atc ata gac Leu Asp Val Met Gln Asp His Phe Ser Arg Ala Ser Ser Ile Ile Asp 175 180 185	759
gag ctc ttc cag gac agg ttc ttc acc cgg gag ccc cag gat acc tac Glu Leu Phe Gln Asp Arg Phe Phe Thr Arg Glu Pro Gln Asp Thr Tyr 190 195 200	807
cac tac ctg ccc ttc agc ctg ccc cac cgg agg cct cac ttc ttc ttt His Tyr Leu Pro Phe Ser Leu Pro His Arg Arg Pro His Phe Phe Phe 205 210 215	855
ccc aag tcc cgc atc gtc cgc agc ttg atg ccc ttc tct ccg tac gag Pro Lys Ser Arg Ile Val Arg Ser Leu Met Pro Phe Ser Pro Tyr Glu 220 225 230 235	903
ccc ctg aac ttc cac gcc atg ttc cag ccc ttc ctt gag atg ata cac Pro Leu Asn Phe His Ala Met Phe Gln Pro Phe Leu Glu Met Ile His 240 245 250	951
gag gct cag cag gcc atg gac atc cac ttc cac agc ccg gcc ttc cag Glu Ala Gln Gln Ala Met Asp Ile His Phe His Ser Pro Ala Phe Gln 255 260 265	999
cac ccg cca aca gaa ttc ata cga gaa ggc gac gat gac cgg act gtg His Pro Pro Thr Glu Phe Ile Arg Glu Gly Asp Asp Asp Arg Thr Val 270 275 280	1047
tgc cgg gag atc cgc cac aac tcc acg ggc tgc ctg cgg atg aag gac Cys Arg Glu Ile Arg His Asn Ser Thr Gly Cys Leu Arg Met Lys Asp 285 290 295	1095
cag tgt gac aag tgc cgg gag atc ttg tct gtg gac tgt tcc acc aac Gln Cys Asp Lys Cys Arg Glu Ile Leu Ser Val Asp Cys Ser Thr Asn 300 305 310 315	1143
aac ccc tcc cag gct aag ctg cgg cgg gag ctc gac gaa tcc ctc cag Asn Pro Ser Gln Ala Lys Leu Arg Arg Glu Leu Asp Glu Ser Leu Gln 320 325 330	1191
gtc gct gag agg ttg acc agg aaa tat aac gag ctg cta aag tcc tac Val Ala Glu Arg Leu Thr Arg Lys Tyr Asn Glu Leu Leu Lys Ser Tyr 335 340 345	1239
cag tgg aag atg ctc aac acc tcc tcc ttg ctg gag cag ctg aac gag Gln Trp Lys Met Leu Asn Thr Ser Ser Leu Leu Glu Gln Leu Asn Glu 350 355 360	1287
cag ttt aac tgg gtg tcc cgg ctg gca aac ctc acg caa ggc gaa gac Gln Phe Asn Trp Val Ser Arg Leu Ala Asn Leu Thr Gln Gly Glu Asp 365 370 375	1335

cag tac tat ctg cgg gtc acc acg gtg gct tcc cac act tct gac tcg 1383
 Gln Tyr Tyr Leu Arg Val Thr Thr Val Ala Ser His Thr Ser Asp Ser
 380 385 390 395

gac gtt cct tcc ggt gtc act gag gtg gtc gtg aag ctc ttt gac tct 1431
 Asp Val Pro Ser Gly Val Thr Glu Val Val Val Lys Leu Phe Asp Ser
 400 405 410

gat ccc atc act gtg acg gtc cct gta gaa gtc tcc agg aag aac cct 1479
 Asp Pro Ile Thr Val Thr Val Pro Val Glu Val Ser Arg Lys Asn Pro
 415 420 425

aaa ttt atg gag acc gtg gcg gag aaa gcg ctg cag gaa tac cgc aaa 1527
 Lys Phe Met Glu Thr Val Ala Glu Lys Ala Leu Gln Glu Tyr Arg Lys
 430 435 440

aag cac cgg gag gag tga gatgtggatg ttgcttttgc acctacgggg gcattctgagt 1585
 Lys His Arg Glu Glu
 445

ccagctcccc ccaagatgag ctgcagcccc ccagagagag ctctgcacgt caccaagtaa 1645
 ccaggc 1651

<210> 14
 <211> 20
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Antisense Oligonucleotide

<400> 14
 gtctttgcac gcctcggtca 20

<210> 15
 <211> 20
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Antisense Oligonucleotide

<400> 15
 attctggagt ctttgcacgc 20

<210> 16
 <211> 20
 <212> DNA
 <213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 16

gtcttcatca tgcctccaat

20

<210> 17

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 17

tctcccaggt cagcagcagc

20

<210> 18

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 18

tctgggtcccc caggacctgc

20

<210> 19

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 19

ggagctcatt gtctgagacc

20

<210> 20

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 20

acttacttcc ctgattggac

20

<210> 21

<211> 20

<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 21
aatttcctta ttgacgtact

20

<210> 22
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 22
gtcttttatct gtttcacccc

20

<210> 23
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 23
gggcacctc tttcttcttc

20

<210> 24
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 24
atttagggca tcctctttct

20

<210> 25
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 25
ttccctggtc tcatttaggg

20

<210> 26
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 26
tgtctctgat tccctggtct 20

<210> 27
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 27
gggagctcct tcagctttgt 20

<210> 28
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 28
cccagagggc catcatggtc 20

<210> 29
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 29
ctcttcccag agggccatca 20

<210> 30
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 30

tcaggcaggg cttacactct

20

<210> 31

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 31

gtgcgtagaa cttcatgcag

20

<210> 32

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 32

ggcggccaac caggcctgag

20

<210> 33

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 33

tggcggccaa ccaggcctga

20

<210> 34

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 34

actcctcaag ctggcggcca

20

<210> 35

<211> 20

<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 35
agtagaaggg cgagctctgg 20

<210> 36
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 36
ttcatccaga agtagaaggg 20

<210> 37
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 37
cagcagggag tcgatgcggt 20

<210> 38
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 38
gctgccggtc gttctccagc 20

<210> 39
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 39
catccagcat gtgcgtctgc 20

<210> 40
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 40
gacatccagc atgtgcgtct 20

<210> 41
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 41
gtggctctgc atgacatcca 20

<210> 42
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 42
aagtggctct gcatgacatc 20

<210> 43
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 43
ctggaagagc tcgtctatga 20

<210> 44
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 44

tgtcctggaa gagctcgtct

20

<210> 45

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 45

gaacctgtcc tggaagagct

20

<210> 46

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 46

ggaaagaaga agtgaggcct

20

<210> 47

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 47

gggcatcaag ctgcggacga

20

<210> 48

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 48

ctcaaggaag ggctggaaca

20

<210> 49

<211> 20

<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 49
tctcaaggaa gggctggaac

20

<210> 50
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 50
tgtatcatct caaggaagg

20

<210> 51
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 51
gctgtggaag tggatgtcca

20

<210> 52
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 52
attctgttgg cgggtgctgg

20

<210> 53
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 53
tatgaattct gttggcgggt

20

<210> 54
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 54
ggatctcccg gcacacagtc 20

<210> 55
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 55
cggatctccc ggcacacagt 20

<210> 56
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 56
gtggagttgt ggcggatctc 20

<210> 57
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 57
gtccttcacg cgcaggcagc 20

<210> 58
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 58

acagtccaca gacaagatct

20

<210> 59

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 59

gagctcccgc cgcagcttag

20

<210> 60

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 60

ggagggattc gtcgagctcc

20

<210> 61

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 61

atcttccact ggtaggactt

20

<210> 62

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 62

tgttgagcat cttccactgg

20

<210> 63

<211> 20

<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 63
agctgctcca gcaaggagga

20

<210> 64
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 64
gctcgttcag ctgctccagc

20

<210> 65
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 65
ttgccagccg ggacacccag

20

<210> 66
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 66
cgcagatagt actggtcttc

20

<210> 67
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 67
accgtggtga cccgcagata

20

<210> 68
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 68
cgagtcagaa gtgtgggaag 20

<210> 69
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 69
gtgatgggat cagagtcaaa 20

<210> 70
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 70
ggagacttct acagggaccg 20

<210> 71
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 71
gccacggtct ccataaattt 20

<210> 72
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 72

gcaaaagcaa catccacatc

20

<210> 73

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 73

tagagtgcag gatccagagc

20

<210> 74

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 74

attagttgca tgcaggagca

20

<210> 75

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 75

agacagtttt attgaattag

20

<210> 76

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 76

cgagatagag ccactgtacg

20

<210> 77

<211> 20

<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 77
tgccaccacc cccgggtgat 20

<210> 78
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 78
gttggtggtg gaacagtcca 20

<210> 79
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 79
tgcttaccgg tgctttttgc 20

<210> 80
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 80
acatctcact cctcccgggtg 20

<210> 81
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 81
gaccctccaa gcgatcagct 20

<210> 82
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 82
aaaaagagga ccctccaagc 20

<210> 83
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 83
tgtgtcccct tttcacctgg 20

<210> 84
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 84
attaccaatg gagcatggca 20

<210> 85
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Antisense Oligonucleotide

<400> 85
caacatggcc aaaccccatg 20

<210> 86
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 86

gcggcaggtc tccaggtctc

20

<210> 87

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 87

ttcccttcgg agagtagaga

20

<210> 88

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 88

tgcttgggaa atgcctgcaa

20

<210> 89

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 89

agctggatgc cagaaaggcc

20

<210> 90

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 90

tggaagtagt ggaagccagg

20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/26235

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 325, 375; 536/23.1, 23.2, 24.3, 24.31, 24.33, 24.5; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)


biosis, medline, caplus, lifesci, embase, uspatfull

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 00/49937 A2 (THE UNIVERSITY OF BRITISH COLUMBIA) 31 August 2000 (31-08-00), see entire document.	1, 2, 4, 5, 11 --- 3, 6-10, 12-20
X --- Y	KANG ET AL. Antisense Oligonucleotide of Clusterin mRNA Induces Apoptotic Cell Death and Prevents Adhesion of Rat ASC- 17D Sertoli Cells. Molecules and Cells. 13 December 1999, Vol. 10, No. 2, pages 193-198, see entire document.	11 -- 1-10, 12-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 23 OCTOBER 2001	Date of mailing of the international search report 12 FEB 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer  KAREN A. LACOURCIERE Telephone No. (703) 306-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/28235

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	MIYAKE ET AL. Antisense TRPM-2 Oligodeoxynucleotides Chemosensitize Human Androgen-independent PC-3 Prostate Cancer Cells Both in Vitro and in Vivo. Clinical Cancer Research. May 2000, Vol. 6, pages 1655-1663, see entire document.	1, 2, 4, 5, 11, 12, 14, 15 -- 3, 6-10, 13, 16-20
X -- Y	MIYAKE ET AL. Testosterone-repressed Prostate Message-2 Is an Antiapoptotic Gene Involved in Progression to Androgen Independence in Prostate Cancer. Cancer Research. 01 January 2000, Vol. 60, pages 170-176, see entire document.	11 --- 1-10, 12-20
X --- Y	MIYAKE ET AL. Acquisition of Chemoresistant Phenotype by Overexpression of the Antiapoptotic Gene Testosterone-repressed Prostate Message-2 in Prostate Cancer Xenograft Models. Cancer Research. 01 May 2000, Vol. 60, pages 2547-2554, see entire document.	11 --- 1-10, 12-20
X - Y	ZWAIN ET AL. Clusterin Protects Granulosa Cells from Apoptotic Cell Death during Follicular Atresia. Experimental Cell Research. March 2000, Vol. 257, pages 101-110, see entire document.	11 --- 1-10, 12-20
X - Y	URBICH ET AL. Laminar Shear Stress Upregulates the Complement-Inhibitory Protein Clusterin A Novel Potent Defense Mechanism Against Complement-Induced Endothelial Cell Activation. November 1999, Vol. 101, pages 352-355, see entire document.	11 --- 1-10, 12-20
X - Y	SENSIBAR ET AL. Prevention of Cell Death Induced by Tumor Necrosis Factor alpha in LNCaP Cells by Overexpression of Sulfated Glycoprotein-2 (Clusterin). Cancer Research. 01 June 1995, Vol. 55, pages 2431-2437, see entire document.	11 --- 1-10, 12-20
Y	US 5,801,154 A (BARACCHINI ET AL.) 01 September 1998 (01-09-98), see especially columns 6-9.	1-20
Y	MILNER ET AL. Selecting effective antisense reagents on combinatorial oligonucleotide arrays. Nature Biotechnology. June 1997, Vol. 15, pages 537-541, see entire document.	1-20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/28235

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):

C07H 21/02, 21/04; A61K 48/00; C12Q 1/68; C12P 19/34; C12N 1/85, 15/86

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/6, 91.1, 325, 375; 536/23.1, 23.2, 24.3, 24.31, 24.33, 24.5; 514/44

THIS PAGE BLANK (USPTO)